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Separation of X- and Y-Chromosome-Bearing Murine Sperm by Free-Flow Electrophoresis: Evaluation of Separation Using PCR

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ABSTRACT—The effectiveness of separation of murine X- and Y-bearing sperm by free-flow electrophoresis was evaluated by the polymerase chain reaction (PCR). The ratio of X- and Y-bearing sperm from cauda epididymis was analyzed before and after free-flow electrophoresis. A Y-chromosome-specific sequence (pY353/B) and an autosomal sequence (myogenin) were used to estimate the ratio between X- and Y-sperm in the separated fractions.

Cauda epididymal mice sperm were separated into two peak fractions under the electric field. Each peak fraction contained sperm of normal shape, however, the motility of the sperm was extremely diminished after separation by electrophoresis. DNA was extracted from 10^4 sperm from each fraction and from the unseparated sperm, and Y-chromosome specific PCR was performed. The PCR experiment revealed that fraction No. 16 (the peak near the cathode) was a Y-sperm rich fraction, whereas fraction No. 22 (the peak near the anode) was a Y-sperm poor one.

These results suggested that murine X- and Y-sperm could be successfully separated by free-flow electrophoresis. Analysis of the chromosome-specific sequence by PCR was demonstrated to be a direct and adequate method to evaluate the separation of X- and Y-sperm.

INTRODUCTION

For the purpose of preselecting the sex of offspring in domestic and laboratory animals as well as in humans, many methods have been developed for separating X- and Y-sperm [1, 2]. Recently, Johnson and collaborators [3–5] succeeded in separating X- and Y-sperm from bull, boar, chinchilla, rabbit, and ram by flow cytometry. However, to distinguish the two fractions, they used the DNA dyes DAPI or H33342 and a high energy laser beam, so the risk of carcinogenesis, DNA damage etc., could not be eliminated. Other methods have been reported to be successful in a limited number of animal species, especially in humans [1].

Attempts to separate sperm by means of galvanization have been made by many workers using various animals [6–9]. We succeeded in separating human sperm by free-flow electorphoresis when examined for the presence or absence of the F-body, a marker for Y-bearing sperm after staining with quinacrine mustard [10–12]. Preliminary results have indicated that various animal sperm also show profiles indicating separation in the electric field. In these animals, however, the F-body test by quinacrine staining can not be used successfully at present. Thus, the evaluation must depend on either chromosome analysis using the zona-free hamster ova [13] or the sex check of offspring.

In recent years, DNA sequences of some specific genes on either X- or Y-chromosome have been determined. These provide useful probes for distiguishing the two sperm populations. In the case of the mouse, Zfy [14], pY353/B [15, 16], and Sry [17] are examples of specific probes for the Y-

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chromosome: Of these pY353/B is highly sensitive because it recognizes a repeating sequence.

In this paper, we shall describe the separation of murine X- and Y-sperm by free-flow electrophoresis and evaluation of purities of the separated fractions by a PCR method for the Y-chromosome-specific repeating sequence.

MATERIALS AND METHODS

Sperm preparation

Sperm were obtained from the cauda epididymis of 4- to 5-month-old ICR male mice and were suspended in a culture dish (35×10 mm, Nunc) containing 0.5 ml of Hanks' balanced salt solution (BSS) and 1% bovine serum albumin (BSA) on a hot plate, followed by preincubation for 15 min at 37°C. The sperm were washed by centrifugation (600×g, 10 min) with the chamber buffer, which contained 0.226 M sucrose, 30 mM $MgSO_4$, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-NaOH buffer, pH 7.2. The supernatant was then discarded, and 0.5 ml of fresh chamber buffer was added to the loosely packed sperm.

Separation of X- and Y-bearing sperm

Separation of murine X- and Y-sperm was carried out using a free-flow electrophoretic apparatus (ACE 710, Hirschmann, FRG) as previously described [10–12]. The separation chamber was composed of two glass plates (4×13 cm) separated by a narrow gap (0.3 mm) in which chamber buffer streamed down with a laminar flow from the top of the chamber to the bottom. While an electric field of 75-V/cm was applied perpendicularly to the buffer stream, the actively motile sperm suspension in a total volume of 0.5 ml was injected continuously into one spot at the top of the chamber. Sperm having different electrophoretic mobilities descended along different paths and were collected into 35 microtubes at the bottom of the chamber. The cell distribution was monitored by measurement of the intensity of scattering light at 520 nm by use of a video camera equipped with a vidicon tube. The internal computer system calculated the cell mass from a series of densitometric tracings, and the results were recorded both on a monitor and on chart paper. Electrophoresis was done at 25°C. Electrode buffer consisted of 100 mM HEPES and 80 mM NaCl, pH 7.0.

DNA extraction from the murine sperm

DNA was extracted from the sperm of each fraction and from the unseparated sperm in capped 1.5-ml polypropylene microcentrifuge tubes. The sperm were suspended in 300 μl of a lysis buffer consisting of 100 mM NaCL, 9 mM EDTA (ethylenediaminetetraacetic acid), 1.5% SDS (sodium dodecyl sulfate), 40 mM DTT (dithiothreitol), 187 μg proteinase K, and 10 mM Tris (tris-hydroxymethyla-minomethane)-HCl buffer, pH 8.0. The mixture was incubated for 90 min at 37°C. Organic extraction was performed as follows; 300 μl of water-saturated phenol/chloroform (1:1) was added to each polypropylene tube. Then the mixture was vortexed 4 times for 20 s each time and centrifuged at 12,000 rpm for 10 min using a TOMY MC-15A centrifuge. The phenol extraction was repeated once and was followed by extraction with Sevag solution (24:1, chloroform: isoamyl alcohol). After the organic extraction, the DNA in the aliquot layer was precipitated overnight at −20°C by the addition of two volumes of 99.9% ethanol. The DNA recovered by centrifugation was resuspended in 70% ethanol for desalting, and then suspended in 60 μl of distilled water.

Standard PCR

About 50 ng of sperm DNA from each fraction obtained by free-flow electrophoresis was amplified enzymatically in 100 μl of a reaction mixture, which include a 1 μM concentration of each primer, 200 μM deoxynucleotide (Pharmacia), 1 unit of *Thermus aquaticus* DNA polymerase (*Taq* polymerase) (Perkin-Elmer Cetus), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.4), and 20 μg/ml gelatin, and was overlaid with mineral oil. PCR was performed in a programmable thermal cycler (Perkin-Elmer Cetus) for 25 or 50 cycles, each consisting of 1 min of denaturation at 94°C, 2 min of annealing at 55°C, and 2 min of polymerization at 72°C. At the end of the cycles, the samples were held at 72°C for 7 min and then cooled.

TABLE 1. List of primers for PCR

Locus	Primer sequence	Length (bp)	Ref.
myogenin	5′-TTACGTCCATCGTGGACAGC-3′ 5′-TGGGCTGGGTGTTAGTCTTA-3′	246	[14]
pY353/B	5′-GAATTCATATATATGACAGACGC-3′ 5′-CCATTCCCTTCAAATATCATACT-3′	102	[15, 16]

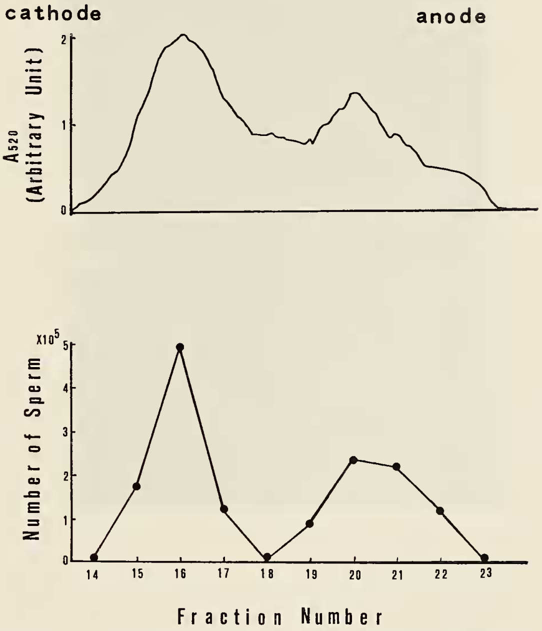

FIG. 1. A typical profile of electrophoretically separated murine sperm from cauda epididymis. The upper shows the densitometric trancing by ACE710 and the lower shows the sperm count of each fraction obtained.

Samples were then either analyzed immediately by agarose gel electrophoresis or frozen overnight at −20°C and analyzed on the following day. Primers used in this experiment are summarized in Table 1.

Gel Electrophoresis

Eighteen μl of the PCR product was mixed with 2 μl of a loading buffer containing 40% sucrose, 0.25% bromphenol blue, and 0.25% xylene cyanol and run at 90 V for 2 h in an agarose gel containing 2.4% SeaKem LE and 1.2% NuSieve in TBE-buffer consisting 45 mM Tris-borate and 1 mM EDTA, pH 8.0.

Densitometric analysis of the PCR products on agarose gel was performed with a DMC-33C densitometer (Toyo Sci. Co. Ltd., Japan).

RESULTS

Separation of sperm by free-flow electrophoresis

Sperm from the cauda epididymis of ICR mice were separated into two distinct peaks by free-flow elctrophoresis. Both peaks migrated toward the anode at pH 7.2. Fig. 1 shows at typical electrophoretic profile obtained with the ACE 710 apparatus and the cell count of each fraction. The two peaks contained virtually the same number of sperm. The separated sperm in both peaks were normally shaped and uncontaminated with other cells but their motility was essentially zero. To evaluate what type of sperm were contained in these two peaks, PCR was performed with the DNA isolated from sperm in each fraction.

PCR of unseparated sperm

As a control experiment, the PCR was performed with DNA extracted from male and female mouse blood and from unseparated sperm. As shown in Fig. 2, both male blood DNA and sperm DNA showed a strong band corresponding to amplified pY353/B. The density of pY353/B was much higher than that of myogenin (an autosomal sequence; lanes 2 and 3 from the left). On the other hand, pY353/B was not detected in female blood DNA, although a good amplification of myogenin was observed (lanes 4 and 5 from the left).

PCR of electrophoretically separated sperm

Sperm fractionated by free-flow electrophoresis

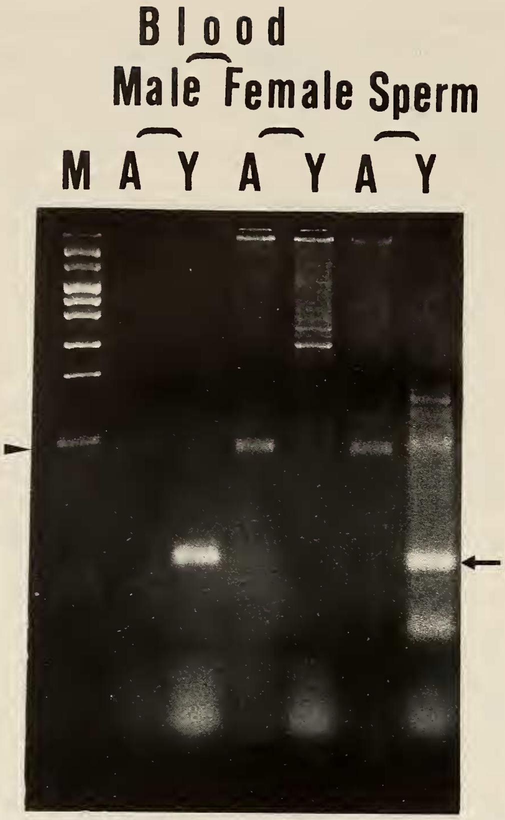

Fig. 2. Ethidium-bromide stained agarose gels of control PCR experiments of pY353/B (Y) and myogenin (A) loci in male blood DNA, female blood DNA, and sperm DNA. M represents molecular size markers of 4,870, 2,016, 1,360, 1,107, 926, 658, 489, 267, 80 bp (pHY marker: Takara Shuzo Co., Ltd., Kyoto, Japan). ←: pY353/B, ◄: myogenin gene.

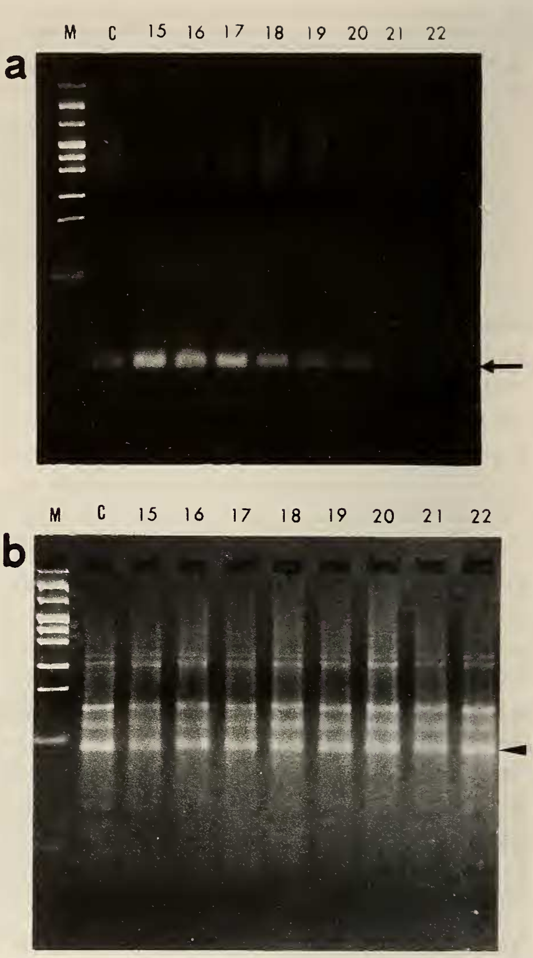

Fig. 3. PCR experiment of unseparated (C) and electrophoretically separated murine sperm. Each number represents the fraction number of free-flow electrophoresis as shown in Fig. 1. For each fraction, DNA was extracted from 10^4 sperm. M represents the same molecular size markers as in Fig. 2. ←: pY353/B, ◄: myogenin gene.

were counted under the microscope and DNA from 10^4 sperm from each fraction was extracted for PCR experiments. A portion of each extracted DNA sample was used for a control experiment for the myogenin gene. Fig. 3 compares the results of the PCR experiments on fractionated and unfractionated sperm. All fractions and control sperm exhibited an equivalent density of the amplified myogenin gene. In other words, all fractions and unfractionated sample contained the same amount of DNA under the present extraction conditions (Fig. 3b). On the other hand, when the Y-chromosome specific sequence pY353/B was amplified by PCR, the fluorescence of etidium-bromide was intense in fractions nearer the cathode. Peak fraction No. 16 exhibited the highest fluorescence of pY353/B, and the fluorescence of the bands gradually diminished toward the anode.

Densitometric analysis of PCR products

The relative value of the density of the pY353/B band in fraction No. 16 was 1.6 times higher than that in the unfractionated sperm, but little difference was seen in the myogenin density. On the other hand, peak fraction No. 22 showed a relative value of 0.2 for the density of the pY353/B band;

and again there was little difference in myogenin band. These results suggested that the cathodic peak fraction No. 16 contained Y-bearing sperm in a high ratio, while the other peak fraction No. 22 did X-bearing sperm by means of the Y-chromosome specific sequence pY353/B.

DISCUSSION

In the present study, the efficiency of sperm separation by means of free-flow electrophoresis was tested by use of the PCR methodology rather than quinacrine staining. The results indicated a successful separation of murine X- and Y-bearing sperm. We clearly demonstrate that DNA analysis is a new and direct method to test the separation of animal sperm. The nonisotopic PCR method used here is more rapid than Southern's technique using specific labeled probes [18]. The former takes about 8 hours, whereas 6 days or more are needed for the latter.

The separation of murine X- and Y-sperm under an electric field was also observed with a zeta-potentialmeter Photal ELS-800 (Otsuka Electronics Co., Tokyo; data not shown). The same was true with human sperm [12]. Furthermore, sperm of other animals such as hamster and mongolian gerbil exhibited similar two-peak profiles (data not shown). This paper is the first description of the electrophoretic separation of X and Y-sperm in a species other than human by means of free-flow electrophoresis. For human sperm, the F-body test by quinacrine staining is the conventional method to detect Y-sperm; but for other animal sperm, the F-body test is not useful and no practical methods, except the measurement of DNA content by flow cytometry has hitherto been available.

The reproducibility of the PCR method was confirmed in a calibration experiment using blood DNA from 50 ng to 1 μg (data not shown). When the amplification of DNA by PCR is performed up to the saturation level, quantitative analysis to estimate the amount of DNA is not appropriate. The present study, however, was carried out at low amplification levels so that the amount of DNA amplified by PCR was roughly proportional to that of applied pY353/B. To eliminate the possibility of artifactral amplification in experiments where only sex chromosome-speicfic sequence were used, the murine autosomal myogenin gene was used to standardize the amplification procedure and the obtained results. Equal portions of each sperm fraction was subjected to pY353/B and myogenin PCR. These reactions were carried out independently to eliminate the possibility that reactions initiated by the two sets of primers would interfere with one another.

Attempts at separating X- and Y-bearing sperm by means of flow cytometry and cell sorting have been made by several workers using various animals, [3–5, 19]. Those have used activation the dyes DAPI or H33342 for staining DNA and a high energy laser beam. Even though the wavelength was relatively long, the risk of hereditary defects such as carcinogenesis could not be excluded. Percoll density gradient centrifugation was effective in obtaining active X-bearing human sperm as the sediment, although the proportion of Y-bearing sperm did not increase in the upper layers [20]. When this techinique was applied to dairy cattle, two thirds of the delivered calves were female, but this result was not statistically significant [1].

Following free-flow electrophoresis, sperm motility was much reduced. Improvements in the separation conditions are needed to maintain sperm activity. However, even in their present condition, separated sperm have a practical application by microinjection into ova. Most importantly, free-flow electrophoresis is performed with intact sperm cells and is applicable not only to human [10–12] but also to murine sperm as demonstrated in this paper. This method therefore to be widely applicable for separating X- and Y-sperm.

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